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Identification of the regulatory genes required for the acid activation of the low pH inducible gene *lpiA* in *Sinorhizobium medicae*

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Declaration

I hereby declare that, unless otherwise stated, the work presented in this thesis is my own.

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Rui, Tian

献给我的父母，为你们对我的的慈爱和包容.

慈母手中线，
游子身上衣。
临行密密缝，
意恐迟迟归。
谁言寸草心，
报得三春晖。

孟郊. 游子吟

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LIST OF PUBLICATIONS

Conference Contributions

1) Rui T, Tiwari, R.P., Bräu, L., O'Hara, G., Howieson, J.G. and Reeve, W.G.(2009): Characterization of the regulatory circuit required for the low pH induction of the adaptive tolerance response protein LpiA in *Sinorhizobium medicae* WSM419. *Proceeding of the 16th International Congress on Nitrogen Fixation.* June 14-19, 2009. Big Sky, Montana.USA

2) Rui T, Tiwari, R.P., Bräu, L., O'Hara, G., Howieson, J.G. and Reeve, W.G. (2009): Regulatory circuit of the acid response protein LpiA in *Sinorhizobium medicae* WSM419**.** *Proceeding of the 15th Australian Nitrogen Fixation Conference.* Nov 8-13, 2009. Margaret River, Western Australia.

3) Rui T, Tiwari, R.P., Bräu, L., O'Hara, G., Howieson, J.G. and Reeve, W.G. (2011). Identification of the regulatory genes required for the acid activation of the low pH inducible gene *lpiA* in *Sinorhizobium medicae*. *Proceeding of the 16th International Congress on Nitrogen Fixation*. Nov 27-Dec 1, 2011. 2011. Fremantle, Western Australia.

It is anticipated that two papers will be published from the material presented in this thesis.

ABBREVATIONS

µ	Micro
A_{260nm}	Absorbance at λ = 260 nm
A_{280nm}	Absorbance at λ = 280 nm
AM3	Antibiotic medium 3
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bp	Base pair
CATB	Hexadecyltrimethyl ammonium bromide
d	Day(s)
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleoside triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
g	Centrifugal force
g	Gram
h	Hour(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilo base pairs
L	Litre
LB	Luria-Bertani
M	Molar
MES	2-(*N*-morpholino) ethanesulfonic acid
mg	Milligram
min	Minute(s)
MOPS	3-(N-morpholino) propanesulfonic acid

MW	Molecular weight
n	Nano
ºC	Degree Celsius
OD_{600nm}	Optical density at λ = 600 nm
ONPG	ortho-Nitrophenyl-β-galactoside
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
s	Second(s)
SDS	Sodium dodecyl sulfate
TAE	Tris, acetic acid EDTA
Tn*5*	Transposon 5
Tris	Tris(hydroxymethyl)aminomethane
TY	Tryptone yeast
UV	Ultra-violet
V	Volt(s)
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside
X-Glc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

ABSTRACT

The study of low pH responsive gene expression in *Sinorhizobium medicae* has identified many genes that are regulated in response to acidic conditions. One of these, the *lpiA* (*l*ow *p*H *i*nducible gene *A*) gene, is acid-activated at least twenty fold by low pH and not by any other stress. The acid-activated expression of *lpiA* has been shown to require functional FsrR (*f*used *s*ensor-*r*egulator). However, even in the absence of FsrR, there is still a 6-fold acid-induction of *lpiA* revealing that other proteins are required for the acid-activation process. Such regulators could be encoded by *tcsA* (*t*wo *c*omponent *s*ensor gene *A*) and *tcrA* (*t*wo *c*omponent *r*egulator gene *A*) which are located upstream of *fsrR* in the *lpiA* gene region. Furthermore, the identification of a potential sigma factor binding motif upstream of *lpiA* suggested the requirement for RpoN (*R*NA *po*lymerase sigma factor for *n*itrogen metabolism) for the transcription process. These regulatory proteins may also be required for the transcription of the *acvB* (*ac*id *v*irulence induced gene *B*) since the DNA sequence contains a *lpiA* stop codon that is coupled to the *acvB* start codon. This would infer that these two genes constitute an operon. It was the aim of this work to therefore investigate the additional regulatory aspects critical for the acid activation of *lpiA* and to identify if the *lpiA* and *acvB* genes are co-transcribed in response to acid.

Mutations were constructed by inserting the CAS-1116 cassette into the *tcsA*, *acvB* and *lpiA* genes of *S. medicae* WSM419 to create the mutants MUR2121, MUR2124 and MUR2127, respectively. Each mutant was constructed by single crossover insertional

inactivation to disrupt the targeted gene and to enable expression to be monitored in each background. In addition, mutants were obtained from previous studies carrying single crossover mutations in *tcrA, rpoN* and *fsrR*.

Phenotypic characterization of *tcsA, tcrA, fsrR, acvB, lpiA* and *rpoN* mutants revealed that these genes are not required for resistance to $ZnCl_2$ (up to 0.7 mM), $CdCl_2$ (up to 0.175 mM), $CuSO_4$ (up to 1.75 mM), NaN_3 (up to 0.175 ml) or ethanol (7%) in comparison to the wild-type WSM419. These genes were also found not to be essential for cell growth in moderately acidic conditions (pH 5.7). However, it was found that a mutation in *lpiA* or *rpoN* slightly decreased the growth rate of the mutants when they were exposed to pH 5.7. Furthermore, in comparison to the wild-type *S. medicae* WSM419, the two mutants were more sensitive to the antibiotic polymyxin B, and were only marginally more sensitive to the antibiotics vancomycin, bacitracin and spectinomycin at pH 5.7. In addition, it was also discovered that the mutation in *rpoN*, but not *lpiA,* caused a symbiotic nitrogen fixation defect for the hosts *Medicago sativa*, *Medicago murex, Medicago polymorpha* and *Medicago truncatula*.

All of the above-mentioned mutants described were constructed by the insertion of a CAS-1116 cassette into the targeted gene. The promoterless *gusA* reporter was used to describe enable transcription to be monitored at the point of insertion. ß-glucuronidase (GUS) determinations at pH 7.0 and pH 5.7 revealed that all of the CAS-1116 induced fusions, including that to *lpiA,* were constitutively expressed in the conditions tested. This finding indicated the unexpected existence of a strong unidentified promoter in the

cassette sequence upstream of *gusA*. Expression of each gene was therefore measured in cells cultured at pH 7.0 and pH 5.7 using the qRT-PCR technique. This technique confirmed the acid-induction of *lpiA* and revealed that *acvB* was also acid-activated to the same extent. This result reinforced the concept that *acvB* was co-transcribed with *lpiA* in *S. medicae*. In contrast, *tcsA, tcrA, fsrR* and *rpoN* were shown to be constitutively transcribed with respect to pH.

To investigate the expression of *lpiA* in each mutant, *gusA* negative backgrounds were constructed. The CAS-1116 cassette (flanked by *loxP* sites) was excised from MUR2121 (*tcsA*:CAS-1116), MUR2124 (*acvB*:CAS-1116), MUR2090 (*tcrA*:CAS-1116), MUR2127 (*lpiA*:CAS-1116) and MUR2088 (*rpoN*:CAS-1116) by Cre-mediated recombination. The resulting *S. medicae* mutants MUR2122, MUR2125, MUR2092, MUR2128, and MUR2093 contained ΔCAS-1116 *loxP* mutations in *tcsA, acvB, tcrA, lpiA* and *rpoN*, respectively. To monitor the expression of *lpiA* in these backgrounds, a plasmid borne *lpiA-gusA* fusion located on pWR220-101H was mobilized into the deletion mutant backgrounds MUR2122, MUR2125, MUR2092, MUR2128 and MUR2093 to create the strains MUR2123, MUR2126, MUR2094, MUR2129 and MUR2095, respectively. The expression of the plasmid borne *lpiA-gusA* fusion was examined in each mutant cultured at low and neutral pH. The fusion was partially induced at pH 5.7 in MUR2092 (~6-fold) and MUR2125 (~10-fold) revealing that TcrA and AcvB both act positively to acid activate *lpiA* transcription. The expression of the *lpiA-gusA* fusion in MUR2122 or MUR2092 was totally abolished revealing that both TcsA and RpoN are essential for *lpiA* expression. This result confirmed a role for RpoN and revealed for the first time a

specific sigma factor that is required for pH regulated gene expression in *S. medicae*. Furthermore, the finding provided credence to the suggestion that the putative RpoN binding motif located upstream of *lpiA* may in fact be the *lpiA* promoter.

To further reinforce this concept, the start site of acid–induced transcription was determined using the 5′ Rapid Amplification of cDNA ends (RACE) technique. The transcription start site for both *lpiA* and *acvB* was found to occur 206 bp upstream of the *lpiA* start codon. The *rpoN* binding site (5′-TG**G**CACG-N4-TTG**C**W-3′) was located adjacent to this identified transcription start site but with the bold G and C in the motif positioned -27 and -14 bases upstream of the transcription start site rather than the expected positioning at -24 and -12. RpoN is known to have a requirement for an enhancer binding protein (EBP) to form the open form complex required for transcriptional activation. A bioinformatics search against the WSM419 genome for proteins carrying a sigma54_interaction domain (Pfam accession number: pfam00158) revealed that the gene product of Smed_5956, located upstream of *tcsA*, is a putative EBP. Future work could investigate whether it is this EBP, or another, that is required for the expression of the *lpiA/acvB* operon.

The results presented reveal that the expression of the low pH induced *lpiA/acvB* operon is regulated in a multifaceted way. Interestingly, weak induction of *lpiA* occurs in the absence of TcrA or FsrR. Since induction was totally abolished in a TcsA mutant, this could infer that TcsA is the cognate sensor for each of these two-component regulatory proteins. In addition, full expression of *lpiA* was shown to occur only if the RpoN sigma

factor was present. This is the first report that has detailed a role for the alternative sigma factor RpoN to regulate the transcription of acid-induced genes. The findings of this thesis have been used to construct a model for the *lpiA* acid-induced regulatory circuit and the implications of this model are discussed.